

ANTI-INFLAMMATORY AND ANTI-NOCICEPTIVE ACTIVITIES OF THE EXTRACTS OF *SARGENTODOXA CUNEATA* AND ITS EFFECTS ON THE MODEL RATS WITH PELVIC INFLAMMATION

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ABSTRACT

An experiment was conducted to determine the concentration of emodin, the main bioactive content of aqueous extracts of *Sargentodoxa cuneata* (SC). In addition, the anti-inflammatory, anti-nociceptive activities and the effects of ESC on pelvic inflammation rats were studied. Twenty-one rodents were randomly divided into a SC group, an indomethacin group and a model control group (n=7 in each group) in an ear edema test, a carrageenin-induced paw edema test, a cotton pellet-induced granuloma formation test, and an acetic acid-induced writhing test, respectively. 60 rats were used for pelvic inflammation test. 50 model rats with pelvic inflammation were established and the serum levels of tumor necrosis factor (TNF- α) and interleukin-6 (IL-6) in each group (n=10) were detected with the Enzyme-Linked ImmunoSorbent Assay (ELISA). Analysis of variance (one-way ANOVA) was employed with 5% significance level (P<0.05) and two-tailed tests were used for all hypothesis tests. The concentration of emodin, a bioactive component in the medicinal plant, was determined by High Performance Liquid Chromatography (HPLC). SC extracts had similar anti-inflammatory and anti-nociceptive activities to indomethacin. ESC significantly decreased the levels of TNF- α and IL-6 of the model rats compared with indomethacin. The content of emodin in ESC was 0.2 mg/g. SC, with its strong anti-inflammatory and anti-nociceptive activities, can be used to treat both acute and chronic inflammation and to relieve the associated pain.

Key words: *Sargentodoxa cuneata*; anti-inflammatory activities; anti-nociceptive activities; pelvic inflammation; emodin.

INTRODUCTION

Sargentodoxa cuneata (SC; family Sargentodoxaceae), as a common Chinese medicinal plant, was traditionally used to remove toxic heat, promote blood circulation, and relieve rheumatic conditions (Li *et al.*, 2005). The aqueous extracts of *Sargentodoxa cuneata* (ESC) has been found to possess the activity against some enterovirus (Guo *et al.*, 2006). Four phenolic glycosides and known phenolic compounds were isolated from the water-soluble constituents of SC. In vitro tests for antimicrobial activity showed phenolic glycosides compounds possess significant activity against two Gram-positive organisms, *Staphylococcus aureus* and *Micrococcus epidermidis* (Chang and Case, 2005).

Researchers found that there was significant correlation between the anti-bacterial activity and the content of emodin in SC (Li *et al.*, 2006). Emodin, an anthraquinone derivative, exhibited anti-inflammatory effect on carrageenan-induced edema in rats (Chang *et al.*,

1996). Emodin has been used to treat many diseases in digestive system for thousands of years. Emodin showed anti-inflammatory activity in 12-Otetradecanoylphorbol-13-acetate model of mouse ear inflammation (Bralley *et al.*, 2008). Emodin, has also shown the anti-cancer effect on several human cancers such as liver cancers and lung cancers (Lai *et al.*, 2009; Su *et al.*, 2005). Ding *et al.* disclosed the mechanism of emodin to treat cholestatic hepatitis via anti-inflammatory pathway (Ding *et al.*, 2008).

Recently, doctors have often applied this in the clinic to treat acute appendicitis, amenorrhea, dysmenorrhea, rheumatic arthralgia, and traumatic swelling and pain (Chang and Case, 2005; Kuang *et al.*, 2005). In clinical practices, the authors found that when treating female patients with pelvic inflammation, SC often led to satisfactory curative effects. The present research was systematically to determine the main bioactive component of aqueous ESC, explore the anti-inflammatory, anti-nociceptive activities and effect of pelvic inflammation of rats.

MATERIALS AND METHODS

Chemicals and plant materials: SC was purchased from Huqing Yutang Pharmaceutical Co., Ltd (Hangzhou, China) identified by the College of Pharmaceutical Sciences, Zhejiang University (Hangzhou, China). Samples in the form of dry plant material were deposited in the herbarium in the College of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou, China. Acetonitrile and methanol (HPLC-grade) were purchased from TEDIA Co. (OH, USA). Acetic acid and perchloric acid (analytical reagent grade) were purchased from Sinapharm Chemical Reagent Co. Ltd (Shanghai, China). Ultra-pure water (made by Millipore, Bedford, MA, USA) with a resistivity of more than 18 M Ω was used for all the preparations. Emodin (Figure 1) and indomethacin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Indomethacin dissolved in 50% ethanol was used as a drug standard. The Enzyme-Linked Immunosorbent Assay (ELISA) kits for tumor necrosis factor (TNF- α) and interleukin-6 (IL-6) were purchased from Boster Biotechnology Ltd., Wuhan, China.

Experimental design: Twenty-one female Sprague-Dawley (SD) rats were used in the carrageenan-induced rat paw edema test, these rats were randomly divided into 3 groups (n=7 in each group): extract of *Sargentodoxa cuneata* (ESC) group, indomethacin group, model control group. 21 Female Imprinting-Control-Region (ICR) mice were used in the ear edema test, cotton pellet-induced granuloma formation test, and acetic acid-induced writhing test, respectively. 21 rats were randomly divided into 3 groups (n=7 in each group): extract of *Sargentodoxa cuneata* (ESC) group, indomethacin group, model control group. 60 rats were used in the study on the effects of the ESC in treating female model rats with pelvic inflammation. 10 rats were randomly taken as the normal control group and the other 50 rats were established as the model rats with pelvic inflammation. The pelvic inflammation rats were randomly divided into three groups: ESC group (n=30); indomethacin group (n=10); model control group (n=10). ESC group, randomly divided into three sub-group (n=10 in each dose group), received orally administered ESC in doses of 70 mg/kg, 210 mg/kg, and 630 mg/kg for 28 consecutive days respectively. The experiment design and layout is shown in figure 2. All the animals were provided by the Laboratory Animal Center of Zhejiang University (Hangzhou, China). All the animals were kept in a room under environmentally controlled conditions of 22 \pm 2 $^{\circ}$ C and a 12h light–12h dark cycle. They were acclimatized at least once a week before starting the experiments. The research was carried out according to

the National Research Council's protocol for the care and use of laboratory animals.

Preparation of the extracts of ESC: 20g of powdered SC was extracted by refluxing it with water followed by filtration. The same extraction procedures were repeated once. The obtained solution was combined and condensed to 1g/ml (wt/vol).

High Performance Liquid Chromatography (HPLC) analysis: The HPLC (Waters model 600E system, Waters, Milford, MA, USA) was equipped with a photodiode array detector (Waters 2996) and an inline-degasser AF (Waters, Milford, MA, USA). A diamonsil C18 column (250mm \times 4.6mm, 5 μ m) from Dikma Technologies (Beijing, China) was equipped with a precolumn (4mm \times 5mm) C18. Acetonitrile (as solvent A) and water/acetic acid (0.8% v/v, pH=6.0; used as solvent B) were used as mobile phase with a linear gradient elution at a flow rate of 1.0 mL/min. The linear gradient elution for ESC was as follows: 0–32 min, linear gradient 5–20% A; 32–35 min, isocratic 20-50% A; 35–40 min, linear gradient 50–85% A. The column temperature was 40 $^{\circ}$ C. The solvents were filtered through a 0.45 μ m Millipore filter and degassed prior to use.

Preparation of stock and working standard solutions: The standard stock solution of emodin was prepared by dissolving 0.03g of emodin in 10 mL of methanol to obtain stock solution concentration of 3.00 mg/ml, which was kept at 4 $^{\circ}$ C. The working standard solutions of emodin with various concentrations of 0.30, 3.00, 30.00, 300.00, and 3000.00 μ g/ml was respectively prepared by diluting the stock solution with methanol. The standard working solutions were kept at 4 $^{\circ}$ C.

Preparation of the samples and the calibration curve: The ESC of 1g/ml was centrifuged at 12,000 rpm for 5 min. The supernatant was filtered through a 0.45 μ m Millipore filter. 20 μ l of each sample solution was analyzed by HPLC. The calibration curve was plotted with the concentrations as X-axis and the peak areas as Y-axis.

Ear edema test in mice: The methods of Brattsand *et al.* (Brattsand *et al.*, 1983) and Young *et al.* (Young and Deyoung, 1981) were used in the test. 21 female mice (weighing 18-22g) were randomly divided into three groups with 7 in each group: ESC group, indomethacin group, model control group. Indomethacin was used as a positive control constituent.

Ear edema was induced by topical application of arachidonic acid (AA) dissolved in acetone to the inner and outer surfaces of both ears with an automatic microliter pipette. 20 μ l of the ESC in dose of 0.01g/ear was administered topically just before the irritant. Comparisons included 20 μ l of indomethacin in dose of 0.5mg/ear (indomethacin group), 20 μ l of 50% ethanol

(model control group). Before and at 0.25h, 0.5h, 1h and 2h after the edema induction, the thickness of each ear was measured by vernier calipers, which was applied near the top of the ear to the cartilaginous ridges. Percent inhibition was calculated: inhibition (%) = $(V_c - V_t) / V_c \times 100\%$, where V_c and V_t respectively represented the average ear thickness of the model-control rat and the rat under drug treatment.

Carrageenan-induced rat paw edema test: The method of Winter *et al.* (Winter *et al.*, 1962) was used in the test. 21 female rats (weighing 140-160g) were randomly divided into three groups with 7 in each group: ESC group, indomethacin group, and model control group.

0.1ml of 1% freshly prepared suspension of carrageenin was administered into the sub-planter region of the right hind paws to lead to the formation of edema in situ due to localized inflammation. The ESC in dose of 70mg/kg was given 1 h prior to carrageenin. Comparisons included indomethacin in dose of 9.97 mg/kg (indomethacin group), saline of 8ml/kg (model control group). Percent inhibition was calculated: inhibition (%) = $(V_c - V_t) / V_c \times 100\%$, where V_c and V_t respectively represented the average paw volume of the model-control rat and the rat under drug treatment.

Cotton pellet-induced granuloma formation tests in mice: The test was conducted as previously described (Yoshida *et al.*, 1994). Twenty-one female mice (weighing 18-22g) were randomly divided into three groups with 7 in each group: ESC group, indomethacin group, and model control group.

The back skin was shaved and disinfected with 75% ethanol. An incision was made in the lumbar region. Subcutaneous tunnels were formed with a blunted forceps. Then, a sterilized cotton pellet weighing 50 ± 1.0 mg was introduced in the groin region of the mouse under light ether anesthesia. The ESC in dose of 6 mg/kg were administered once daily throughout the 7 day experimental period. Comparisons included indomethacin in dose of 1.44mg/kg (indomethacin group), saline of 8ml/kg (model control group). Twenty-four hours after the treatment ended, the animals were sacrificed and the cotton pellets were excised, which were then dried until the weight remained constant. The increase of the pellet weight was considered as granuloma tissue deposit.

Acetic acid induced writhing test in mice: The method of Ghia *et al.* (Ghia *et al.*, 2004) was used in the test. 21 female mice (weighing 18-22g) were randomly divided into three groups with 7 in each group: The ESC in dose of 6 mg/kg was orally administered 0.5h prior to acetic acid. Comparisons included indomethacin in dose of 1.44mg/kg (indomethacin group), and saline of 8ml/kg (model control group).

A writhing response was produced by injection of an aqueous solution of 0.6% acetic acid in a volume of

0.1 ml/10g body weight into the peritoneal cavity and the animals were then placed in a transparent plastic box. Test drugs and saline were orally administered 0.5h before the acetic acid injection. Writhes number, a response consisting of contraction of an abdominal wall, pelvic rotation followed by hind limb extension, was counted during continuous observation for 15 min beginning from 5 min after the injection of acetic acid.

The effects of ESC in treating female model rats with pelvic inflammation: 60 female SD rats (weighing 140-160g) were used in the test. 10 rats were randomly taken as the normal control group and the other 50 rats were established as the model rats with pelvic inflammation. The model rats' pelvic inflammation was established by injecting 0.08ml of 20% phenol mucilage into the right uterus of the rats to induce a pathological condition similar to pelvic inflammation. The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). 20% phenol mucilage was obtained by combining 8ml phenol with 32ml of 1% carboxymethylcellulose sodium mucilage. After 15 days, the model was established. 50 rats were randomly divided into three groups: ESC group (n=30); indomethacin group (n=10); model control group (n=10). ESC group, was randomly divided into three sub-group (n=10 in each dose group), received orally administered ESC in doses of 70 mg/kg, 210 mg/kg, and 630 mg/kg for 28 consecutive days respectively.

Indomethacin group received orally administered indomethacin at 9.97mg/kg for 28 consecutive days; Model control group received orally administered saline at 8ml/kg for 28 consecutive days; Normal control group received orally administered saline at 8ml/kg for 28 consecutive days. When the treatment ended, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The serum sample taken from liver portal vein was used to detect the levels of tumor necrosis TNF- α and IL-6 with ELISA.

Statistical analysis: Results were analyzed by an independent statistician using computer software, namely, Statistical Package for Social Sciences (SPSS 15.0 for Windows). Analysis of variance (one-way ANOVA) was employed in the ear edema test, carrageenan-induced rat paw edema test, granuloma weight of cotton pellet-induced granuloma formation tests, acetic acid-induced writhing test and in comparing the changes of TNF- α and IL-6 of various groups. A 5% significance level ($P < 0.05$) and two-tailed tests were used for all hypothesis tests.

RESULTS AND DISCUSSION

The concentration determination of emodin in the ESC extracts: The regression equation was calculated in the form of $Y = aX + b$, where Y represents the peak area and X represents the content of emodin. The regression

equation of emodin is $Y=129295X+11541.6$. The linear range is from $0.30\mu\text{g/ml}$ to $3000.00\mu\text{g/ml}$. The precision of intra-day and inter-day of the method was 0.8% and 1.1%. The emodin recovery was from 98.4% to 100.5%, with RSD less than 1.6%. Emodin content of ESC is from $10.84\mu\text{g/g}$ to $15.23\mu\text{g/g}$. The HPLC profile of ESC is shown in Figure 3.

Ear edema in mice: The results of the ear edema test in mice are shown in Figure 4. Both ESC and indomethacin groups showed significant inhibition on the ear edema. Indomethacin had a stronger anti-inflammatory effect at 0.25h. However, from 0.5h to 2h there was no significant difference between ESC and indomethacin groups.

Carrageenan-induced rat paw edema test in rats: The results of the carrageenan-induced rat paw edema test are shown in Figure 5. Both ESC and indomethacin groups showed a significant reduction of the carrageenan-induced paw edema volume compared with the model control group. ESC had a lower inhibitory effect from 1h to 2h. While from 3h to 5h, there existed no significant difference between ESC and indomethacin. ESC has more potent inhibition effect on 5h compared with indomethacin.

Cotton pellet-induced granuloma formation tests in rats: The results of the chronic-inflammatory test with cotton pellet are shown in Table 1. The extracts of ESC and indomethacin both demonstrated significant inhibitory activity on the weight of granuloma. ESC had significantly inhibitory effects on the granuloma formation than indomethacin.

Table 1 Comparison of the results of the cotton pellet-induced granuloma formation tests

Group	Dry granuloma weight (mg/mg cotton)	Granuloma inhibition (%)
Model control	6.23 ± 0.17	-
ESC	$3.07\pm 0.11^{*,\#}$	41
Indomethacin	$3.78\pm 0.09^*$	34

The significant difference was set at $*p < 0.05$, compared with the model control group; $\# p < 0.05$, compared with the indomethacin group.

Table 2 Comparison of the results of acetic acid induced writhing tests

Group	Number of writhes	Inhibition of writhing response (%)
Model control	50.0 ± 5.2	-
ESC	$26.0\pm 6.8^{*,\#}$	48
Indomethacin	$21.3\pm 4.4^*$	58

The significant difference was set at $*p < 0.05$, compared with the model control group; $\# p < 0.05$, compared with the indomethacin group.

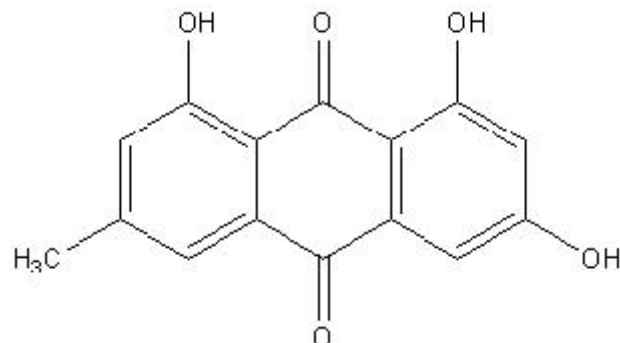


Figure 1. The Structure of emodin

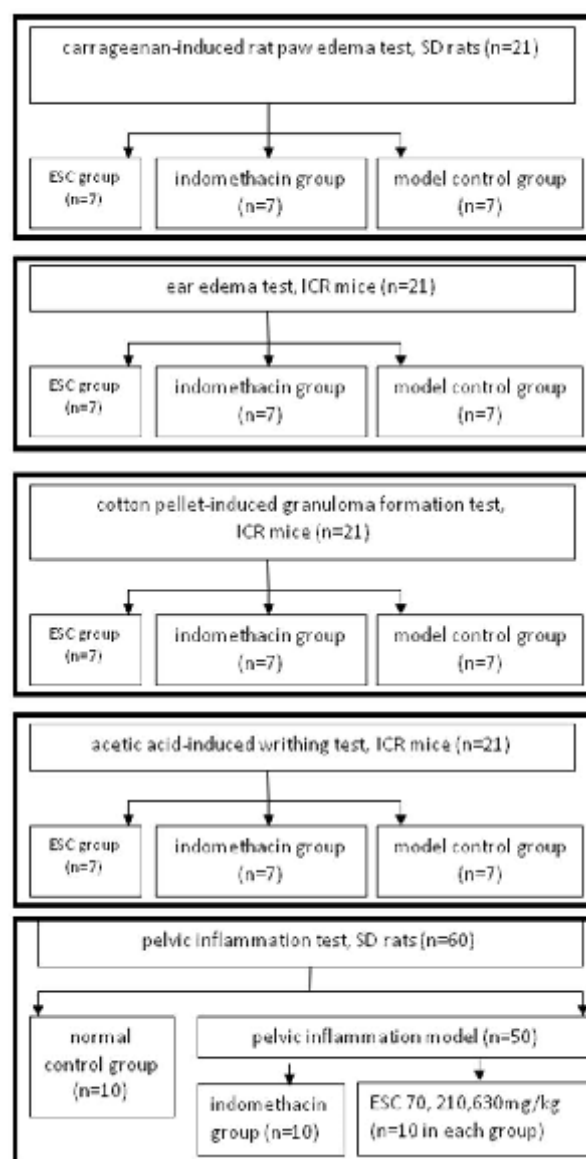


Figure 2. The experiment design and layout

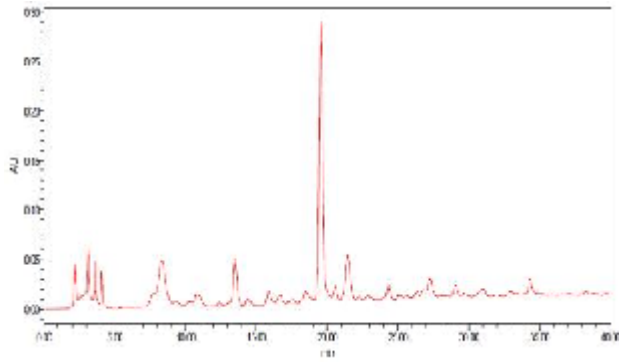


Figure 3. The HPLC chromatograms of the extracts of *Sargentodoxa cuneata* (ESC) 1: emodin

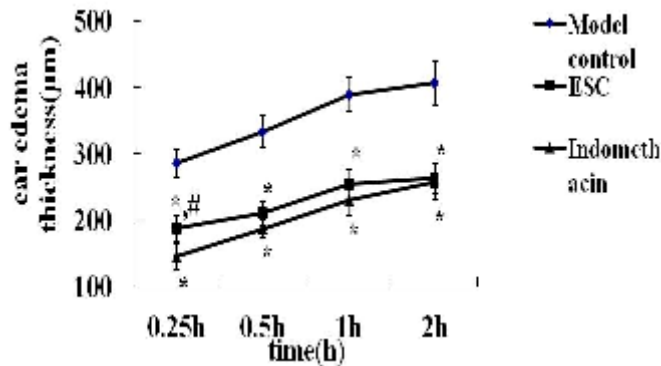


Figure 4. Comparison of the results of ear edema tests. The significant difference was set at $*p < 0.05$, compared with the model control group; $#p < 0.05$, compared with the indomethacin group.

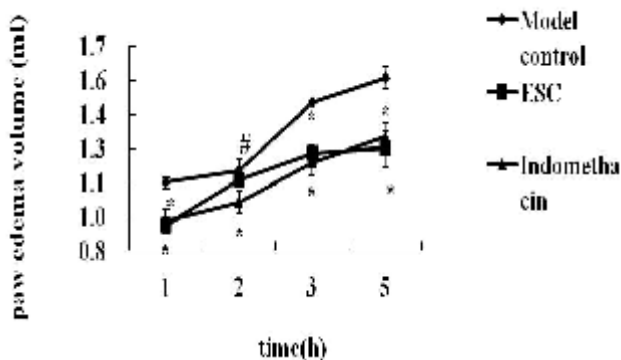


Figure 5. Comparison of the results of carrageenin-induced paw edema tests. The significant difference was set at $*p < 0.05$, compared with the model control group; $#p < 0.05$, compared with the indomethacin group.

Acetic acid induced writhing test in mice: The results of the acetic acid induced writhing test in mice are shown in Table 2. Both the extracts of ESC and indomethacin had inhibitory activity on the writhing response. There

existed significant difference between them. The inhibitory activity of ESC was lower than indomethacin.

Comparison of the serum levels of TNF- α and IL-6: The serum levels of TNF- α and IL-6 in ESC and indomethacin groups were all significantly lower than those of the model control group. ESC extracts significantly inhibited TNF- α and IL-6 to a greater extent than indomethacin. The serum levels of TNF- α of all the ESC groups were significantly lower than the indomethacin group (Figure 6). The serum level of IL-6 in the ESC sub-group in dose of 630mg/kg was significantly lower than that of indomethacin group (Figure 7).

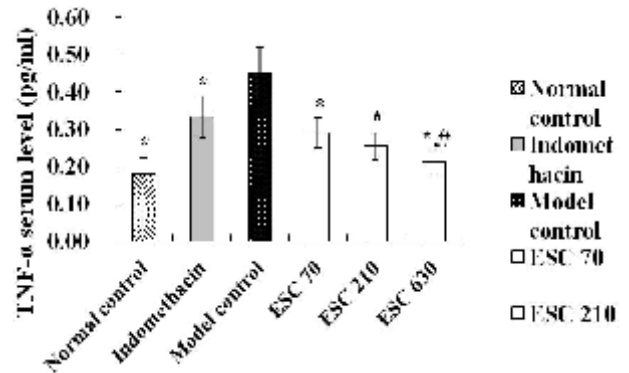


Figure 6. Comparison of the serum levels of TNF- α . The significant difference was set at $*p < 0.05$, compared with the model control group; $#p < 0.05$, compared with the indomethacin group.

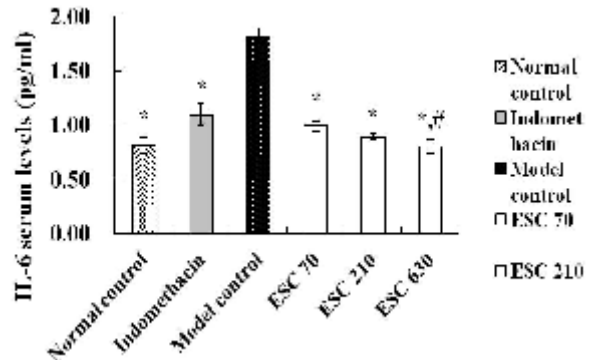


Figure 7. Comparison of the serum levels of IL-6. The significant difference was set at $*p < 0.05$, compared with the model control group; $#p < 0.05$, compared with the indomethacin group.

In the present research, the model rats with pelvic inflammation were established by injecting 0.08ml of 20% phenol mucilage into the right uterus of the rats to induce a pathological condition similar to pelvic inflammation (Zhou et al., 2008). The method has been

widely used to establish animal models with pelvic inflammation since 1980, which is thought to be simple in manipulation, effective at inducing pelvic inflammation, and similar to the human in pathology (Miescher *et al.*, 1985). As TNF- α and IL-6 are both important cytokines during the process of pelvic inflammation, they were used to compare the curative effects of the various treatments on the model rats with pelvic inflammation in the present research.

Emodin is the typical constituent of ESC. Emodin has been known to exist in at least 17 plant families worldwide, and has been investigated for anti-inflammatory, anti-fungal, anti-parasitic, antioxidant, immunosuppressive, and anti-ulcer activities (Chang *et al.*, 1996; Gao *et al.*, 2011). Emodin is often determined as a quantity control constituent of ESC. Liu *et al.* (Liu *et al.*, 2007) determined emodin content in Danning tablet by HPLC coupled with electrospray tandem mass spectrometry (ESI-MS) and ultraviolet detector (UV). Li *et al.* (Li *et al.*, 2006) explored the correlation between emodin content of ESC tables and anti-bacterial activity. Emodin showed the anti-bacterial effect on experimental strains of *Staphylococcus aureus* and *Bacillus subtilis*. They found that there was difference among the content of the secondary metabolites and anti-bacterial activity of ESC tables from different areas and batches.

Quantitative assessment of emodin in the plant has been conducted with HPLC. However, other compounds in ESC with anti-inflammatory and anti-nociceptive effects are indistinct. Further research should be conducted on the other compounds in the plant. It is concluded that ESC, with its strong anti-inflammatory and anti-nociceptive activities, can be used to treat both acute and chronic inflammation and to relieve the associated pain.

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